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Point Mutations in the Varicella-Zoster Virus DNA Polymerase Gene Confers Resistance to Foscarnet and Slow Growth Phenotype

Bertrand Visse, Jean-Marie Huraux, and Anne-Marie Fillet*
Laboratoire de Virologie, CERVI, Hôpital Pitié-Salpêtrière, Paris, France

Seven independent laboratory mutants were derived from seven distinct wild-type varicella-zoster virus (VZV) isolates after exposure to increasing concentrations of foscarnet (PFA) and were found to be resistant to this drug. Single base changes resulting in amino acid substitutions were observed in the nucleotide sequence of the DNA polymerase gene of each PFA-resistant mutant. The mutations were found to occur within the domain II (Arg-665 → Gly for strains vrMOR and vrVER; Val-666 → Leu for vrLEB; Gln-692 → Arg for vrOLI) and domain III (Arg-806 → Ser for vrABD ; Leu-809 → Ser for vrALI and vrCHA) of DNA polymerase gene. In addition, the PFA-resistant mutants exhibited a phenotype characterized by slow growth, the strains showing a marked delay in immediate-early antigen plaque formation compared with the wild-type VZV from which they were derived. These results may have implications for successful isolation and characterization of PFA-resistant strains from clinical samples containing mixed viral populations. ***J. Med. Virol. 59:84–90, 1999.***

KEY WORDS: varicella-zoster virus; foscarnet; mutant laboratory; resistance; DNA polymerase

INTRODUCTION

Acyclovir is the most widely prescribed antiviral drug for the management of recurrent and occasionally disseminated varicella-zoster virus (VZV) infection in patients with AIDS. The isolation of acyclovir-resistant VZV strains in this population was becoming an emerging clinical problem [Pahwa et al., 1988; Jacobson et al., 1990; Linnemann et al., 1990; Snoeck et al., 1993; Talarico et al., 1993; Boivin et al., 1994; Fillet et al., 1998], although since the beginning of HAART therapy the incidence of herpes viruses resistance to antiviral therapy is decreasing. Foscarnet (phosphonoformic acid, PFA) is an analog of pyrophosphate (PPi), a product of polymerization of nucleic acids. Unlike acyclovir, it does not require activation by any viral enzyme, but rather directly inhibits DNA polymerase. The mechanism of inhibition is not the competition with deoxynucleoside triphosphate [Oberg, 1983]. Rather, it appears that PFA acts by binding to the site normally occupied by pyrophosphate [Crumpacker, 1992]. PFA has been shown to be a potentially effective and tolerable antiviral agent for patients with acyclovir-resistant VZV infection [Safrin et al., 1991]. Most studies of VZV sensitivity to PFA have yielded conflicting results between clinical and in vitro resistance [Safrin et al., 1991; Bendel et al., 1993]. One case of clinical resistance to PFA with corresponding virus resistance in vitro in an AIDS patient has been described [Fillet et al., 1995], and a single point mutation that entailed a change from a glutamic acid to lysine at position 512 in the DNA polymerase gene (ORF28) associated with PFA-resistance was recently shown [Visse et al., 1998].

Based on studies of the DNA polymerase gene of herpes simplex virus (HSV), several conserved regions of this gene among herpes viruses designated I through VI have been described [Wong et al., 1988]. The sequence analysis of several HSV or human cytomegalovirus (HCMV) polymerase mutants revealed that mutations conferring resistance to antiviral drugs that mimic and/or compete with the natural deoxynucleoside triphosphate (dNTP) or PPi substrates for enzyme binding are distributed within the regions I to VII and in the region A. This suggests that these conserved regions are involved directly or indirectly in recognition and binding of deoxynucleotides and PPi [Coen et al., 1991]. Despite the common assumption that a single mutation in the DNA polymerase gene could be

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*Correspondence to: Anne-Marie Fillet, Laboratoire de Virologie, CERVI, Hôpital de la Pitié-Salpêtrière, 75651 Paris Cedex 13, France.

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responsible for resistance [Larder et al., 1987; Gibbs et al., 1988; Hwang et al., 1992; Lurain et al., 1992; Sullivan et al., 1993; Baldanti et al., 1996], to date no experimental data elucidating the molecular basis of VZV resistance to PFA have been presented.

In an attempt to approach this question, in this study seven PFA-resistant VZV mutants derived from clinical isolates verified to be genetically unrelated by using DNA typing have been obtained after exposure to the drug. These mutants have been characterized with respect to their susceptibility to PFA in vitro; the nucleotide sequence of their DNA polymerase gene and their growth phenotype, and the modification of PFA susceptibility of each mutant in conserved regions of the DNA polymerase gene has been mapped. All PFA-resistant mutants were found to have a single amino acid change in the domains II (for four PFA-resistant mutants) or III (for three PFA-resistant mutants) of the DNA polymerase gene and exhibited slower viral growth in cell cultures. These results permitted the identification of critical amino acids involved in the interaction with PFA.

MATERIALS AND METHODS

Cells, Virus Isolation and Plaque Purification

Human lung carcinoma cells (A549) and human diploid lung fibroblasts (MRC-5, BioMérieux, Lyon, France) were propagated in Eagle's minimum essential medium (MEM) (Gibco BRL, Paisley, Scotland) supplemented with 5% fetal calf serum, vancomycin (20 μg/ml), amykacin (20 μg/ml), and L-glutamine (300 μg/ml).

The VZV isolates ABD, ALI, CHA, LEB, MOR, OLI, and VER were recovered from seven otherwise healthy children with chickenpox and were plaque-purified three times by end-point dilutions. These isolates and the strain OKA were characterized as PFA-susceptible viruses.

Isolation of PFA-Resistant Mutants

The PFA-resistant mutants were obtained by exposure of purified wild-type strains at increasing concentrations of PFA. A A549 cell line flask monolayer was inoculated with each strain and maintained in medium containing 16 μM PFA. Inoculated flasks were examined daily and passed when a VZV-characteristic cytopathic effect (CPE) was observed. Each isolate was then exposed to increasing concentrations of PFA from 16 μM to 66 μM. Isolates which showed 100% CPE at 66 μM were passed and exposed to increasing concentrations of PFA at increments up to 1330 μM. The PFA-resistant isolates were characterized as showing 100% CPE in the presence of 1330 μM PFA.

Antiviral Susceptibility Measured by a Late-Antigen Synthesis Reduction Assay

The drug susceptibility of VZV isolates was determined by using a late-antigen synthesis reduction assay [Fillet et al., 1998]. Briefly, MRC-5 monolayer cells in a 24-well plate were inoculated with four ten-fold dilutions of VZV-infected cells. Eight control wells were overlaid with MEM medium containing 10% fetal calf serum and no PFA. The remaining wells were overlaid with a medium containing a range of PFA concentrations (66, 166, 500, and 1,000 μM). The 50% inhibitory concentration (IC50) was determined as the concentration of antiviral agent which reduced the number of late-antigen synthesis foci by 50% as compared to those produced in the control wells. The test was performed in duplicate for each wild-type and mutant strain and in parallel with the reference strain OKA.

Slow-Growth Phenotype Analysis

OKA, wild-type viruses and PFA-resistant mutant strains were used to infect confluent MRC-5 culture grown in a 24-well plate.

To analyse viral growth, infected cells were fixed at 1, 2, 4, and 8 days postinfection (p.i.) and stained with the monoclonal antibody 2013 (Argéne Biosoft, Varilhes France) to the major immediate early antigen (IEA) p63. This staining permitted the detection of single infected cells and IEA-expressing foci. To determine the titer of the infected-cell-associated virus, cultures were stained with the same monoclonal antibody 1U1 (Argéne Biosoft, Varilhes France) for detection of late antigen (M. Harzic, personal communication) at 2 days p.i. as used for antiviral susceptibility.

DNA Typing of VZV Wild-Type Viruses and PFA-Resistant Mutants

Two fragments were amplified and shown to cover the regions spanning genes 38 to 43 (G38-G43, 11,4 kb) and 54 to 60 (G54-G60, 7,7 kb) [Takayama et al., 1996], respectively.

Amplification involved a hot-start procedure and the Expand Long Template PCR System (Boehringer Mannheim, France) was used as instructed by the manufacturer. DNA samples were amplified for 40 cycles, each cycle consisting of denaturing at 92°C for 10 seconds, primer annealing at 65°C for 30 seconds, and chain elongation at 68°C for 10 minutes. In the first cycle, samples were denatured at 92°C for 2 minutes, in the last 20 cycles, the elongation step was increased for more yield of 20 second for each cycle, and in the last cycle, the extension step was increased to 15 minutes. PCR products were purified with a Wizard PCR Preps kit (Promega, Madison, WI) and used for digestion with Taq I, Fok I, Alu I, and Rsa I from G38-G43 and with Hinf I, Hae III, and MspI (Appligene, Illkirch, France) from G54-G60 amplified products.

DNA Polymerase Gene Amplification and Sequencing

The entire ORF 28 gene coding for the VZV DNA polymerase gene was amplified in a single step PCR using the primer pair POL1-POL2 as previously described [Visse et al., 1998].

Sequencing was undertaken directly on PCR products purified with Wizard PCR Preps kit. Primers for sequencing, stained with 5'-Texas-Red, were synthesized (Genset SA, Paris, France) from published VZV

sequences at 200 to 300 bp intervals [Davison et al., 1986]. Sequences were determined by the dideoxynucleotide-chain termination method [Sanger et al., 1977] using the Thermo-Sequenase core sequencing kit with 7-deaza-dGTP (RPN 2440 Amersham, France) and an automatic sequencer (DNA Sequencer 725; Vistra DNA System, Amersham France). The sequences were aligned and overlapped by computer software (GenWorks 2.5.1., IntelliGenetics, Inc., Mountain View, CA).

RESULTS

Isolation of PFA-Resistant Mutants

The seven VZV isolates were submitted to increasing concentrations of PFA as described in Materials and Methods. In each case, a PFA-resistant mutant exhibiting 100% CPE in the presence of 1330 μM of PFA was obtained. The seven mutants were designated vrABD, vrALI, vrCHA, vrLEB, vrMOR, vrOLI, and vrVER.

Antiviral Susceptibility Testing

The IC50 values of PFA for OKA and wild-type strains were in a range of 50 to 114 μM (Table I).

The antiviral susceptibility profiles of PFA-resistant mutants showed a marked increase of the IC50 for all the PFA-resistant mutant strains as compared to the IC50 of wild-type strains (Table I). The 50% sensitivity index (SI50) was determined to be the ratio of the VZV and OKA strains. For wild-type strains, the SI50 ranged from 0.9 to 1.3 and for PFA-resistant mutants, from 4.8 to 7.2 (Table I).

TABLE I. Susceptibility to PFA of Wild-Type and Corresponding PFA-Resistant Mutants VZV Strains

Wild type			Mutants		
Strain	IC_{50} (μM)	SI_{50}[b]	Strain	IC_{50} (μM)	SI_{50}
OKA	72 ± 16[a]				
ABD	83 ± 3	1.1	vrABD	434 ± 25	6
ALI	69 ± 19	0.9	vrALI	409 ± 12	5.6
CHA	98 ± 16	1.3	vrCHA	517 ± 14	7.2
LEB	81 ± 2	1.1	vrLEB	368 ± 9	5.1
MOR	93	1.3	vrMOR	352 ± 2	4.8
OLI	96 ± 2	1.3	vrOLI	346 ± 7	4.8
VER	67 ± 6	0.9	vrVER	351 ± 19	4.8

[a]Mean values from duplicates experiments ± SD.
[b]SI_{50} (50% sensitivity index) = VZV strain IC_{50}/OKA IC_{50}. Each SI_{50} was determinated with mean values from OKA IC_{50} obtained during the same antiviral susceptibility.

Slow-Growth Phenotype Analysis

It had previously been observed that all VZV strains resistant to PFA show a delay in ECP formation in cell culture compared with each wild-type sensitive strains. To verify the hypothesis that the PFA-resistant mutants could be associated with a slower replication, the time course of the IEA plaque formation of OKA, MOR, OLI, VER and vrMOR, vrOLI, vrVER viruses was studied on confluent MRC-5 cultures. At 1 day p.i. PFA-resistant mutant-infected cultures showed a smaller focus stained with monoclonal antibody 1U1 (late antigen) (not shown) or monoclonal antibody 2013 (IEA antigen) than those observed in OKA or wild-type infected cells (Fig. 1). At 4 days p.i. all PFA-resistant mutants showed small IEA plaques while OKA and wild-type infected cells showed large IEA plaques. At 8 days p.i., each PFA-resistant mutant showed a smaller plaque formation than those of OKA or the wild-type strains, suggesting a slower replication of the mutant strains with respect to the strain from which they were derived.

RLFP Analysis of Wild-Type and Mutant Viruses

The comparative DNA restriction analysis of distinct genome regions performed on the wild-type viruses showed that most strains were unrelated (Table II). The wild-type strains CHA and OLI have a restriction fragment pattern identical to that of Dumas strain which was taken as the reference. The five other isolates showed restriction patterns distinct from the Dumas strain and each other (Table II).

Regarding the PFA-resistant mutants each one exhibited a restriction fragment pattern identical to its wild-type counterpart (not shown).

DNA Polymerase Analysis

The nucleotide sequence of the entire DNA polymerase gene from OKA and each wild-type strain was therefore determined and showed the variability of this gene. Comparison of sequencing data with the published Dumas sequence [Davison et al., 1986] showed a Gly-to-Cys and a Ser-to-Gly change at position 186 and 863, three silent mutations of the OKA strain, only one silent mutation of ABD strain, a Gln-to-Lys change in position 298 of LEB strain, a Ser-to-Gly change in position 863, two silent mutations of the MOR strain, and a Leu-to-Met change at position 1095 of the VER strain (Table III). For the ALI, CHA, and OLI strains, no mutations were found in comparison to the Dumas sequence.

The nucleotide sequence from each PFA-resistant mutant showed the same mutations as those observed from each wild-type strain in comparison with the Dumas sequence. Comparison of sequencing data with each wild-type sequences showed that each PFA-resistant mutant has another mutation with an Arg-to-Ser change at position 806 of vrABD, an Leu-to-Ser change at position 809 of vrALI and vrCHA, an Val-to-Leu change at position 666 of vrLEB, an Arg-to-Gly change at position 665 of vrMOR and vrVER, and a Gln-to-Arg change at position 692 of vrOLI.

Each mutant polymerase gene was found to contain a single base change resulting in an amino acid substitution in domain II (strains vrMOR, vrVER, vrLEB, and vrOLI) and in domain III (strains vrABD, vrALI, and vrCHA) (Fig. 2).

DISCUSSION

The isolation and characterization of seven PFA-resistant mutants of VZV are described. On the basis of the PFA mechanism of action and data already available for HSV PAA-(phosphonoacetic acid) and PFA-

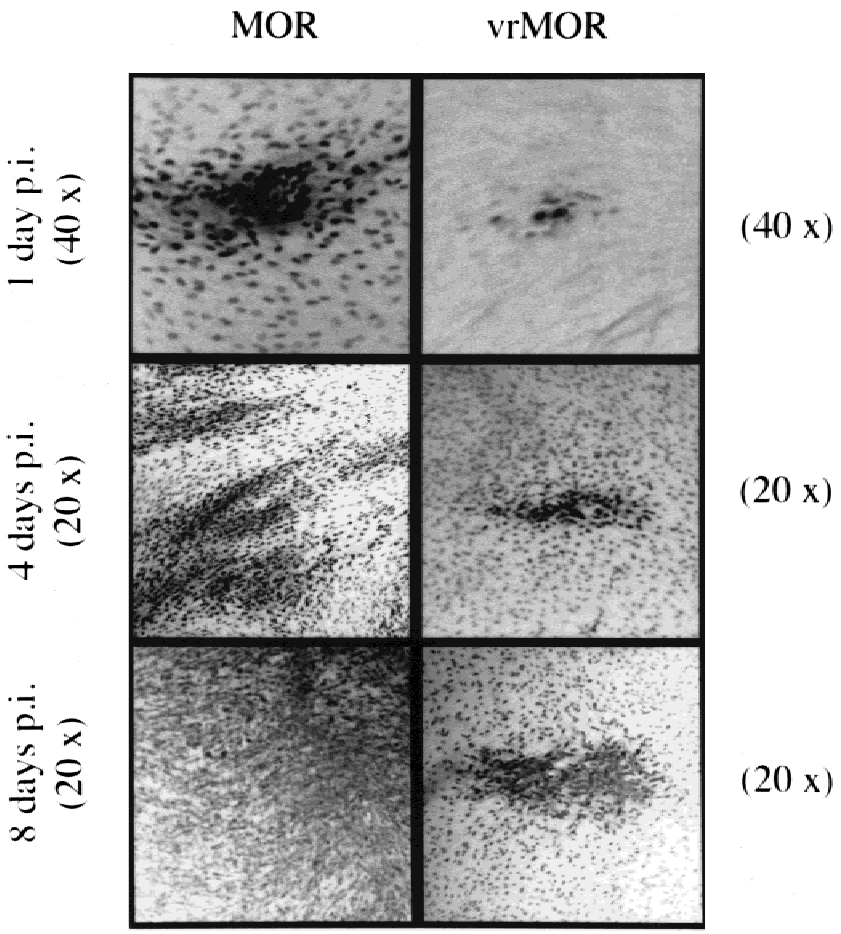

Fig. 1. Slow growth phenotype analysis. MOR and vrMOR- infected MRC5 monolayers at 1, 4, and 8 days p.i. stained with monoclonal antibody 2013 by immunoperoxydase technique. For each row, the magnification is given in parentheses.

resistant laboratory strains [Larder et al., 1987; Gibbs et al., 1988], and HCMV PFA-resistant strains [Baldanti et al., 1996], VZV PFA-resistant strains were predicted to have DNA polymerase mutations.

Using sequencing of each wild-type and derived PFA-resistant strain, among all amino acid mutations of DNA polymerase, only those which were located in conserved regions were found to be present in PFA-resistant mutants. Five different amino acid substitutions associated with resistance to PFA were thus identified. These mutations were located in domain II (vrMOR and vrVER, Arg-665 → Gly; vrLEB, Val-666 → Leu; and vrOLI, Gln-692 → Arg) and in domain III (vrABD, Arg-806 → Ser; vrALI and vrCHA, Leu-809 → Ser) (Fig. 2). All the mutations of PFA-resistant mutants located outside the conserved domains were also detected in the wild-type strains from which they were derived and were never situated between domains IV and I. These results suggest that these mutations are not implicated in PFA-resistance and represent a spontaneous genetic polymorphism.

Domain II is one of the most conserved regions of the herpes virus DNA polymerases [Larder et al., 1987; Teo et al., 1991] and has been suggested as a strong candidate for the pyrophosphate binding site [Larder et al., 1987]. On the basis of genetic analysis of HSV and HCMV resistant strains [Larder et al., 1987; Gibbs et al., 1988; Baldanti et al., 1996], it was observed that amino acid substitutions which confer PFA- and/or PAA-resistance are clustered in this region. PFA-resistance to the HCMV mutant was demonstrated by Thr to Ala change at position 700 [Baldanti et al.,

TABLE II. Restriction Fragment Length Polymorphism (RFLP) Found in VZV Wild-Type Isolate and PFA-Resistant Mutants

VZV strain	RFLP[a]						
	Genes 38 to 43				Genes 54 to 60		
	Taq I	Fok I	Alu I	Rsa I	Hinf I	Hae III	Msp I
ABD	0	+	0	+	0	0	0
ALI	0	0	0	0	0	–	0
CHA	0	0	0	0	0	0	0
LEB	0	0	0	0	+	0	0
MOR	+	0	–	0	0	0	+
OLI	0	0	0	0	0	0	0
VER	0	+	0	0	0	0	+

[a]Restriction patterns compared to those of VZV Dumas strain taken as reference: 0, restriction site identical to that of the Dumas strain; +, gain of an additional restriction site; –, loss of a restriction site.

TABLE III. Nucleotide and Amino Acid Changes DNA Polymerase of Wild-Type Isolates and PFA-Resistant Mutants in Comparison With Dumas Sequence [Davison et al., 1986]

Wild type			Mutants		
Strain	Nucleotide change	Amino acid change	Strain	Nucleotide change	Amino acid change
OKA	GGT 556 → TGT[a]	Gly 186 → Cys			
	CTG 1102 → TTG	—[b]			
	ACC 1812 → ACT	—			
	AGT 2587 → GGT	Ser 863 → Gly			
	TTG 2697 → TTA	—			
ABD	CCG 1248 → CCA	—	vrABD	CCG 1248 → CCA	—
				CGT 2416 → AGT	**Arg 806 → Ser**[c]
ALI	—	—	vrALI	TTA 2426 → TCA	**Leu 809 → Ser**
CHA	—	—	vrCHA	TTA 2426 → TCA	**Leu 809 → Ser**
LEB	CAA 892 → AAA	Gln 298 → Lys	vrLEB	CAA 892 → AAA	Gln 298 → Lys
				GTT 1996 → CTT	**Val 666 → Leu**
MOR	CGC 78 → CGT	—	vrMOR	CGC 78 → CGT	—
	TAT 1632 → TAC	—		TAT 1632 → TAC	—
	AGT 2587 → GGT	Ser 863 → Gly		AGG 1993 → GGG	**Arg 665 → Gly**
				AGT 2587 → GGT	Ser 863 → Gly
OLI	—	—	vrOLI	CAG 2075 → CGG	**Gln 692 → Arg**
VER	CTG 3283 → ATG	Leu 1095 → Met	vrVER	AGG 1993 → GGG	**Arg 665 → Gly**
				CTG 3283 → ATG	Leu 1095 → Met

[a]Nucleotides changes are underlined.
[b]No change.
[c]Amino acid substitution associated with resistance to PFA are in bold type.

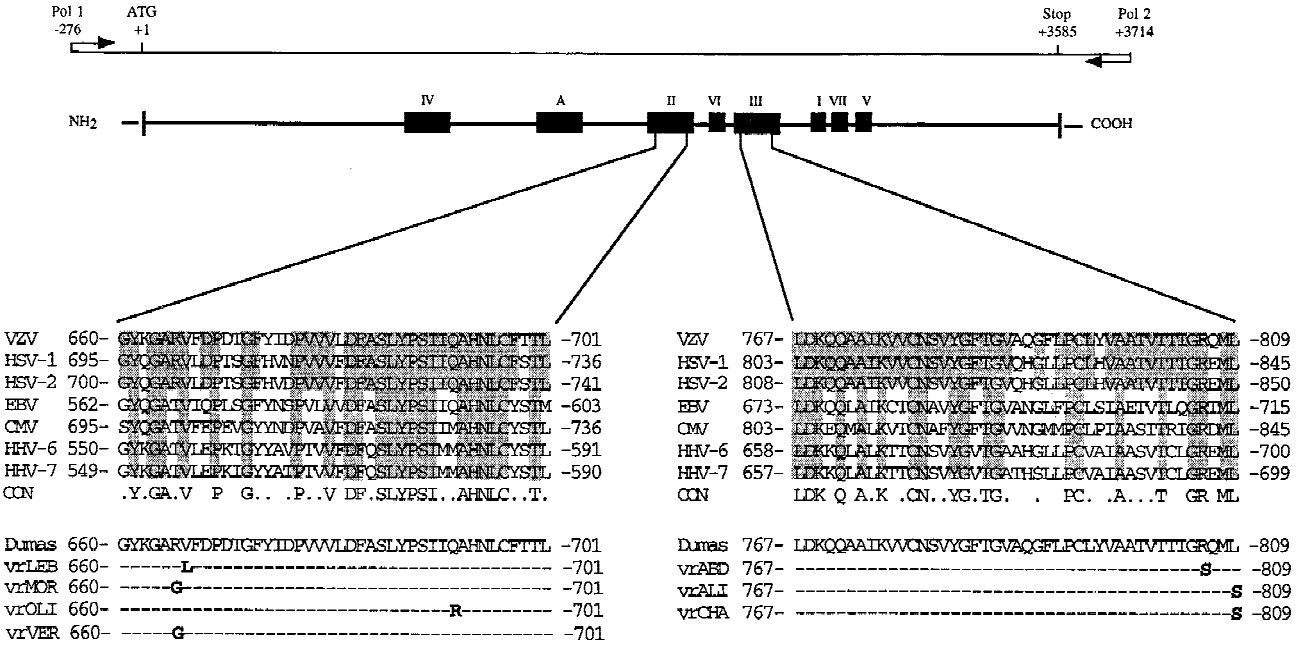

Fig. 2. Structure of the VZV DNA polymerase gene and primer positions. Solid boxes represent the conserved domains of sequence conserved between DNA polymerase [Gibbs et al., 1988; Wong et al., 1988; Hwang et al., 1992]. Below is shown the amino acid sequence of domains II and III from a number of herpesvirus DNA polymerase including : HSV-1, HSV-2, EBV, CMV, HHV-6 [Teo et al., 1991], and HHV-7 [Nicholas, 1996]. Amino acids identical to the VZV sequence are shaded and on consensus (CON), point indicates amino acid relatively conserved in all herpes viruses. The amino acid sequences of fragments of vrLEB, vrMOR, vrOLI, and vrVER domain II and vrABD, vrALI, and vrCHA domain III containing the mutations conferring PFA resistance (in boldface) are shown.

1996], and the mutation in the strain PFAr1 that confers resistance to PAA and ACV is a relatively nonconservative Arg to Gly change at position 700 on the HSV DNA polymerase [Gibbs et al., 1988]. For two PFA-resistant mutants (vrMOR and vrVER) the Arg to Gly change shown by Gibbs et al. [Gibbs et al., 1988] at position 665 on the HSV DNA polymerase was also found (Fig. 2). On the vrLEB mutant, a Val to Leu change was seen at position 666, a position highly conserved in all herpes viruses and close to the amino acid clearly implicated in PFA-resistance [Gibbs et al., 1988; Baldanti et al., 1996]. Three other mutations were documented and responsible for PFA- or PAA-resistance in HSV and HCMV. Two PFA-resistance

HCMV strains were found to have a Val to Met change at position 715 [Baldanti et al., 1996]. Larder et al. [1987] had found, in a HSV PAA-resistant strain, an Ala to Val change at position 719, and another mutation has been documented at position 724 with a Ser to Asp change in PFA- and PAA-resistant HSV strain [Larder et al., 1987; Gibbs et al., 1988]. The last mutation observed in domain II was Glu to Arg at position 692 from vrOLI strain. This mutation was situated in the middle of 13 amino acids that are conserved in all herpes viruses, one of which is implicated in HSV PFA-resistance to PFAr5 strain [Gibbs et al., 1988].

Three of the PFA-resistant mutants have their mutation lying within domain III which has been suggested as a nucleoside-binding site [Crumpacker, 1992]. This region has been implicated in many cases of HSV PAA- and/or acyclovir-resistance [Larder et al., 1987; Gibbs et al., 1988]. PAA-resistance to a HSV mutant was demonstrated by Arg to Ser change at position 842 [Gibbs et al., 1988], the same position mutation that for vrABD also has an Arg to Ser substitution at position 806 of the DNA polymerase gene (Fig. 2). Arg is found at this position in all herpes virus DNA polymerases. We have found two PFA-resistant mutants whose mutation was a Leu to Ser at position 809 from vrALI and vrCHA. Leu also is found at this position in all of the herpesvirus DNA polymerases.

The genetic characterization of a PFA-resistant strain of VZV isolated from a patient with AIDS has shown the emergence of a mutation at position 512, which is a glutamic acid substitution to a lysine [Visse et al., 1998]. Rather than being located in domain II or III of the DNA polymerase this mutation was between domain IV and A. While results concerning localisation of mutation in the DNA polymerase gene associated with PFA resistance in domain II and III for laboratory mutants and between domain IV and A for clinical isolate conflict, one hypothesis could be proposed. VZV laboratory mutants with mutation in domain II or III of the DNA polymerase are not easily isolated because of their slow growth.

A slower replication of the PFA-resistant strains with respect to the reference strain OKA and each wild-type from which they were derived were also observed. We have demonstrated slower growth in culture cells for only three VZV PFA-resistants (vrMOR, vrOLI, and vrVER) whose mutations are located in domain II. However, the same phenomenon of slower replication was observed in all seven PFA-resistant strains. Baldanti et al. [1996] have described a DNA polymerase mutation located in domain II of HCMV PFA resistant isolate and have demonstrated that the mutation is responsible for both PFA resistance and slow-growth phenotype. The domain II of the VZV DNA polymerase is involved in PFA recognition and binding, and this domain could play an important role in the DNA polymerase function.

It is concluded that single amino acid changes in domain II or III of the VZV DNA polymerase of mutants may confer PFA resistance. The slower growth of this VZV strain may have implications for the successful isolation, propagation and characterization of PFA-resistant strains from clinical samples containing mixed viral populations.

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